claim 134: page 43, lines 4-9. Page 53, line 35 to page 54, line 1. Claims 121-134 do not involve any issue of new matter. Therefore, entry of this amendment is respectfully requested.

Applicants would like to thank Examiner Duffy for discussing the subject application with Spencer Schneider of the undersigned attorney's office during a June 1, 2001 telephone conference. During the telephone conference, Examiner Duffy indicated that she would examine composition claims, such as those presented herein.

January 17, 2001 Communication

In the July 17, 2001 Communication, the Examiner stated that the proposed reply filed on October 16, 2000 is not responsive to the prior Office Action because of the following alleged ommissions or matters. The Examiner stated that the amendment to the claims constitutes a change of invention for the following reasons. The Examiner stated that the previous claims were examined in light of applicants' amendment to recite an oligosaccharide portion of a ganglioside conjugated through a ceramide-derived carbon thus requiring a portion of the ganglioside to be present. The Examiner stated that the previous claims were considered in light of this specific linkage and the presence of the ganglioside. The Examiner stated that the now claimed invention does not require that any part of the ceramide portion of the ganglioside to be present and has apparently changed the linkage. The Examiner stated that the presently claimed invention provided by applicants' amendment does not reflect gangliosides or linkage through ceramide-derived carbons. The Examiner stated that since applicants have already received an action on the merits for an alleged different claimed invention (linking the oligosaccharide portion of a ganglioside

conjugated through a ceramide derived carbon), the amended claims drawn to any oligosaccharide conjugated to Keyhole Limpet Hemocyanin constitutes a change of invention. The Examiner stated that such changes are not permissible mid-prosecution.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject pplication have hereinabove canceled claims 102-120 without disclaimer or prejudice to applicants' right to pursue the subject matter of these claims in a later-filed application and added new claims 121-134. Applicants respectfully point out that newly added claim 121 recites a "composition which comprises: a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) an immunogenic protein-based carrier; b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, wherein the ganglioside derivative is a derivative of a ganglioside selected from the group consisting of GM2, GM3, GD2, GD3, GD3 lactone, O-acetyl GD3 and GT3; and wherein the immunogenic protein-based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin; wherein in the conjugate the ganglioside derivative is **conjugated to the immunogenic protein-based carrier through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative** [Emphasis added]. Applicants contend that this amendment

obviates the objection raised in the January 17, 2001 Communication and respectfully request that the Examiner enter the amendment and examine the invention of claim s 121-134.

Composition claims

The Examiner stated that the previously submitted composition claims 57-70 and newly submitted claims 98-101 are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: the compositions as claimed are distinct because they can be used in a materially different process such as linked to a column for purification of cross reactive antibodies, in an in vitro method to study immune responses or in an in vitro method to generate monoclonal antibodies. The Examiner stated that since applicant has received an action on the merits for the originally presented methods invention, this invention has been constructively elected by original presentation for prosecution on the merits and accordingly, claims 57-70 are withdrawn from consideration as being directed to a non-elected invention. The Examiner stated that applicants assert that the claims do not define patentably distinct inventions and the groups should be rejoined. Applicants remarks are not persuasive because the compositions can be used in materially different methods and are distinct as set forth above, the restriction is proper and made final. The Examiner stated that the inventions are related as disclosed but distinct as claimed, and restriction is proper. The Examiner stated that there is no requirement that the invention be both independent and distinct as asserted by applicants, and cited MPEP 803 "the inventions must be independent or distinct as claimed." The Examiner moreover stated that there is an undue search and examination burden since the

inventions are classified differently necessitating a different search of at least the US Patents. The Examiner further stated applicants may not petition under 37 CFR 1.29(b) to rejoin because no restriction has been made in the present application due to actions by the applicant, as clearly evidenced by the cancellation of compositions in preliminary amendments A mailed June 7, 1995 and B, mailed November 15, 1995.

In response, applicants note that the examiner agreed in the June 1, 2001 telephone conference to examine either "composition of matter" of claims, or "methods of use" claims, but not both Accordingly, without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have previously canceled claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-134. Applicants respectfully point out that newly added claims 121-134 are composition of matter claims. Applicants contend that this amendment obviates the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Priority

The Examiner stated that applicants recently filed a request for a corrected filing receipt indicating that the instant application was a 371. The examiner stated that the instant application was not filed under 35 USC 371 and that it is not now nor ever has been accorded 371 status. The Examiner that correction is required in response to this office action.

In response, applicants respectfully point out that they filed a

Third Communication Requesting Corrected Filing Receipt on April 12, 2001.

Obviousness type double patenting

The Examiner provisionally rejected claims 71-97 under the judicially created doctrine of obviousness type double patenting as being unpatentable over claims 65-71 and 77 of copending Application No. 08/477,097. The Examiner stated although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species drawn to GM2 or GM3 claimed in the copending application would anticipate the instant genus method claims. The Examiner stated applicants' argue that the provisional rejection should be allowed to drop and that the instant claims be allowed to issue, pursuant to MPEP section 804. The Examiner stated since the instant claims are not allowable, the provisional double patenting rejection is maintained for reasons already made of record.

The Examiner provisionally rejected claims 71-97 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 66-72 of copending Application No. 08/475,784. The Examiner stated although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the copending application would anticipate the instant genus method claims. The Examiner stated applicants' argue that the provisional rejection should be allowed to drop and that the

instant claims be allowed to issue, pursuant to MPEP section 804. The Examiner stated since the instant claims are not allowable, the provisional double patenting rejection is maintained for reasons already made of record.

The Examiner provisionally rejected claims 71-97 under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 80-86 and 92-96 of copending Application No. 08/196,154. The Examiner stated although the conflicting claims are not identical, they are not patentably distinct from each other because they all claim conjugating proteins to gangliosides through the ceramide portion and thus the particular method species claimed in the copending application would anticipate the instant genus method claims. The Examiner stated applicants argue that the provisional rejection should be allowed to drop and that the instant claims be allowed to issue, pursuant to MPEP section 804. The Examiner stated since the instant claims are not allowable, the provisional double patenting rejection is maintained for reasons already made of record.

In response, applicants without conceding the correctness of the above rejection but to expedite prosecution of the subject application have hereinabove canceled claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-134. Applicants contend that these amendments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 71-97 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention is maintained for reasons made of record for claims 44 and 46-56 in Paper No. 13, mailed 4-1-98 and reasons herein. The Examiner stated as to claims 72-77 and 89-97, Applicants arguments and evidence have been carefully evaluated but is not persuasive to remove the rejections of record in regard to prevention of cancer, and prevention of relapse of cancer. The Examiner stated that applicants provide Zhong et al (Cancer Research, 58:2844-2849, 1998) and contend that Zhong et al protects against syngeneic tumor challenge and eliminates micro metastases. The Examiner stated this evidence is not persuasive for instant prevention of cancer or prevention of relapse for the reasons set forth below. The Examiner stated first, the composition did not prevent cancer as is claimed (see claims 72 and dependent claims). The Examiner stated as seen on page 2846, while the composition comprising GD2 conjugated to KLH via the ceramide double bond to aldehyde by ozonolysis and attachment of KLH by reductive amination in the presence of cyanoborohydride in combination with QS-21 extended survival, however, mice still died. The Examiner stated prevention of cancer is not still enabled even with the composition comprising GD2 conjugated to KLH via the ceramide double bond to aldehyde by ozonolysis and attachment of KLH by reductive amination in the presence of cyanoborohydride in combination with QS-21. The Examiner stated in regard to prevention, the tumor challenge was limited to a single type of cancer and administered by intravenous challenge. The Examiner stated the claims are broadly drawn to

prevention of any cancer and is not limited to the specific conjugate and lymphoma treated. The Examiner stated in contrast to solid tumors, the intravenous compartment would be expected to have any antibodies present in high concentration. The Examiner stated this situation is unlike the majority of cancers which are not present in the intravenous compartment. The Examiner stated applicants have not provided evidence that solid tumors can be either treated or prevented by administration of any compositions as is claimed. The Examiner stated Curti et al (Critical Reviews in Oncology/Hematology, 14:29-39, 1993) teach the numerous physical barriers to drug delivery in solid tumors. The Examiner stated applicants have not taught these types of cancers can be prevented prior to the onset of cancer or any relapse also prevented. The Examiner stated Zhong et al teach that the administration of the composition after a reduced tumor challenge did not provide a statistically significant difference (see page 2847, column 1, second full paragraph) between the control and the composition. The Examiner stated thus, prevention is not enabled and relapses are not enabled since the Zhong et al article does not enable prevention of relapse, because the primary tumor is still present and relapse can occur at the primary tumor site. The Examiner stated all relapses are not due to metastacies. The Examiner stated thus, the specification as originally filed does not enable the prevention of any type of cancer.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have previously canceled claims 57-101 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application, hereinabove canceled

claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-134. Applicants respectfully point out that newly added claims 121-134 relate to compositions of matter. Applicants also point out that claims 121-134 do not recite methods of preventing cancer. Applicants contend that these amendments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Claims 71-97

The Examiner stated that as to claims 71-97, the claims are still not enabled for derivatives of KLH wherein the derivative comprises KLH linked to an immunological adjuvant. The Examiner stated the specification does not teach how to link or conjugate immunological adjuvants, such as cytokines, non-ionic block copolymer or monophospholipid A, to the KLH moiety. The Examiner stated the specification does not teach that the compositions so made function to boost the immune response toward the oligosaccharide moiety, rather than the KLH moiety to which the adjuvant is attached or are useful in any manner contemplated by the specification. The Examiner stated the specification does not reference any art accepted method of making said derivatives. The Examiner stated Pierce (U.S. Patent No. 5,616,477) teaches that many materials have been shown to have adjuvant activity, however, such chemical coupling involves harsh treatment and often results in destruction of a portion of the antigen and reduced immunogenicity (column 1, lines 30-40). The Examiner stated applicants have not taught how to make a ganglioside or oligosaccharide portion of the ganglioside retains its immunogenicity at any level, especially at that level which is

required to treat or prevent cancer as is instantly claimed. The Examiner stated moreover, the art teaches that the antigen *per se* is directly coupled to the immunological adjuvant. The Examiner stated the claim requires that the adjuvant be linked through the KLH carrier protein and thus the adjuvant would be expected to increase the immunogenicity of the KLH rather than the ganglioside or oligosaccharide attached thereto. The Examiner stated in view of the absence of showing of the ability of such compositions to prevent of cancer or prevent relapses of cancer, the absence of any teaching of how to make these derivatives suitable for use in the claimed methods, one skilled in the art would be forced into undue experimentation to make such derivatives and use them in the methods of the invention as are now claimed. The Examiner stated in the absence of further guidance on how to make a ganglioside-KLH derivative, it would require undue experimentation to predictably and reproducibly make the compositions and use the compositions in the claimed.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have previously canceled claims 57-101 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application, hereinabove canceled claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-134. Newly added claims 121-134 do not recite "derivatives of Keyhole Limpet Hemocyanin." Applicants contend that these amendments obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. §103(a)

The Examiner rejected claims 71-88 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al (Cancer Research, 49:7045-7050, 1989) in view of Irie et al (U.S. Patent No. 4,557,931, published December 10, 1985) and Ritter et al (Cancer Biology, 2:401-409, 1991) is maintained and reiterated below. The Examiner stated that Livingston et al (Cancer Research, 49:7045-7050, 1989) teach a composition administered to melanoma patients for stimulating the production of antibodies directed to a carbohydrate epitope on the ganglioside, GM2 (p 7046-7048). The Examiner stated Livingston et al teach that the GM2 is administered in conjunction with an adjuvant, Bacillus Calmette-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline (p 7048, column 1, paragraph 3 and paragraph bridging p 7046-47). The Examiner stated Livingston et al teach that the melanoma recurrence was delayed in patients developing GM2 antibodies after vaccination (p 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated Livingston et al teach that more patents produced IgM antibodies that IgG antibodies to the GM2 (9 7047 paragraph bridging columns 1-2). The Examiner stated Livingston et al also teach the gangliosides GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (p 7045, column 1, paragraph 2). The Examiner stated Livingston et al do not teach the conjugation of the GM2 vaccine with Keyhole Limpet Hemocyanin (KLH) through the ceramide portion of the ganglioside or use of any of the other gangliosides in a method to induce an immune response or cancer treatment.

The Examiner stated that Irie et al teach conjugation of

ganglioside GM2 to a non-toxic protein carrier, such as albumin, using ozonolysis (column 5, see B., lines 19-68) which conjugates a the GM2 through the ceramide portion. The Examiner stated Irie et al teach that the fatty acid of ceramide maybe removed leaving shpingosine and thus the coupling takes place through the amine group of the sphingosine moiety (column 2, lines 64-69). The Examiner stated Irie et al teach that the conjugated GM2 can be used as a vaccine to stimulate an immune response and raise the anti-GM2 titer in mammals (column 2). The Examiner stated Irie et al differ by not conjugating the GM2 to KLH.

The Examiner stated that Ritter et al (Cancer Biology, 2:401-409, 1991) teach that the IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (p 406, paragraph 1). The Examiner stated Ritter et al discloses the advantage of using an IgG antibody response (versus IgM) against ganglioside is that IgG a) has a higher affinity; b) is better able to penetrate solid tissues; c) is able to mediated antibody-dependent cell-mediated cytotoxicity; and d) is generally detectable in the serum for longer periods after immunization. The Examiner stated it would have been *prima facie* obvious to one of ordinary skill in the art to modify the GM2-albumin ceramide conjugate of Irie et al by substituting KLH for albumin and to substitute the resulting GM2-KLH ceramide conjugate for the GM2 in the immunization composition of Livingston et al for active immunization for generating antibody response for melanoma treatment because Irie et al teach that the GM2 conjugated through the ceramide (sphingosine) portion can be used as a vaccine to simulate an immune response and raise the anti-GM2 titer in mammals

and Ritter et al teach that the IgG responses to gangliosides may be increased by covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (p 406, paragraph 1) and Ritter et al discloses the advantages of generating and IgG as opposed to an IgM antibody response and optimization of the dosage, route of administration and number of sites to administer the composition as combined above is well within the skill of the art.

The Examiner stated applicants allege that the references neither individually or combined teach the invention as is now claimed. The Examiner stated this is not persuasive. The Examiner stated applicant's arguments fail to comply with 37 CFR. 1.111(b) because they amount to a general allegation that the claims patentably distinguishes them from the references. Applicant's arguments do not comply with 37 CFR 1.111(c) because they do not clearly point out the patentable novelty which he or she thinks the claims present in view of the state of the art disclosed by the references cited or the objections made. The Examiner stated further, they do not show how the amendments avoid such references or objections.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants contend that Livingston et al (Cancer Research, 49:7045-7050, 1989) in view of Irie et al (U.S. Patent No. 4,557,931) and Ritter et al (Cancer Biology, 2:401-409, 1991) does not render obvious the claimed invention.

Applicants point out that newly added claim 121 recites a "composition which comprises: a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an

altered ceramide portion comprising a sphingosine base, to ii) an immunogenic protein-based carrier; b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, wherein the ganglioside derivative is a derivative of a ganglioside selected from the group consisting of GM2, GM3, GD2, GD3, GD3 lactone, O-acetyl GD3 and GT3; and wherein the immunogenic protein-based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin; wherein in the conjugate **the ganglioside derivative is conjugated to the immunogenic protein-based carrier through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative**."

First, the Examiner acknowledges that the primary reference, i.e. Livingston et al. Cancer Research 1989, ("Livingston 1989") does not teach conjugation of GM2 with Keyhole Limpet Hemocyanin through the ceramide portion of the ganglioside (see January 11, 2000 Office Action, page 8).

To compensate for the lack of such disclosure, the Examiner relies relies on two references: Irie et al (U.S. Patent No. 4,557,931); and Ritter et al (Cancer Biology 1991). However, applicants submit that neither of these references supplies what it missing from the primary reference.

The Examiner relies on two section of Irie: (1) column 5, lines 19-

68; and (2) column 2, lines 64-69. However, neither of these sections of Irie teach the conjugation as recited in the claimed invention.

Applicants point out that Irie et al. at column 5, lines 19-68, discloses conjugation through the **oligosaccharide** of the ganglioside. In contrast, the applicants' claims recite that the conjugation is through a C-4 carbon of the sphingosine base of the **ceramide** portion of the ganglioside derivative. In addition, applicants' claims recite that the ganglioside derivative comprises an **unaltered oligosaccharide part** and an altered ceramide portion. Accordingly, with respect to this portion of Irie, not only does not disclose the claimed conjugation, it teaches away from it by teaching that the conjugation is in the oligosaccharide portion and not the ceramide portion of the ganglioside.

The second portion of Irie to which the Examiner cites (i.e. column 2, lines 64-69) contains two references with respect to the conjugation.

The first reference (column 2, lines 63-65) states that "the entire ceramide portion of GM2 may be removed (in which event the oligosaccharide of GM2 is coupled through the **glucose** moiety)" [emphasis added]. Applicants respectfully point out that such conjugation is through the **glucose** moiety and not through the ceramide. Therefore, this portion of Irie does not disclose the conjugation as recited in the claims.

The second reference (column 2, lines 65-68) states that "the fatty acid of the ceramide may be removed leaving sphingosine (in which

event the oligosacharide of GM2 is coupled through the **amine group of the sphingosine moiety**)" [emphasis added]. In contrast, the conjugation as recited in the claims states that it is "through a **C-4 carbon of the sphingosine base** of the ceramide portion of the ganglioside derivative." Since Irie teaches that the conjugation is through an **amine** group of the sphingosine, while the claims recite that the conjugation is though a **C-4 carbon** of the sphingosine, Irie does not disclose the conjugation as recited in the claims. Nor does Irie suggest conjugation through the C-4 carbon.

Accordingly, Irie et al does not supply what is missing from the primary reference with respect to the conjugation.

The second reference upon which the Examiner relies is Ritter 1991. Ritter 1991 discloses on page 406, column 1 two approaches for augmenting the immunogenicity of gangliosides in a mouse, and states that only one of these approaches is capable of inducing consistent IgG antibodies to gangliosides in the mouse. Ritter 1991 describes this approach as covalently attaching gangliosides to foreign carrier proteins such as KLH.

Although Ritter 1991 refers to the conjugation of GM2 to KLH, there is no description of the chemical nature of the conjugate or of how to make the conjugate. Thus, Ritter 1991 neither discloses anything conjugated through the ceramide, nor enables making any such conjugate. Applicants respectfully direct the Examiner's attention to the highlighted portion of claim 121 above relating to the conjugation, which recites "the ganglioside derivative is conjugated to the immunogenic protein-based carrier through a C-4 carbon of the sphingosine base of the ceramide portion of the

ganglioside derivative." The Examiner's indication that one skilled in the art would interpret Ritter 1991 to involve ceramide conjugation is only speculation. Based on Ritter 1991, one skilled in the art would not understand that the linkage would be through the ceramide. Therefore, Ritter 1991 does not supply what is missing from either the primary reference (i.e. Livingston et al.) or the other secondary reference (i.e. Irie et al.)

Therefore, Livingston et al in view of Irie et al and Ritter et al does not render obvious the claimed invention. Applicants contend that these amendments and remark obviates the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 103(a)

The Examiner rejected claims 71-88 under 35 U.S.C. 103(a) as being unpatentable over Livingston et al. (Cancer Research, 149:7045-7050, 1989) in view of Ritter et al. (Seminars in Cancer Biology, 2:401-409, 1991), Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol, 182:32-43, 1990), Kensil et al. (The Journal of Immunology, 146(2):431-437, 1991), and Marciani et al. (Vaccine, 9:89-96, 1991) and Uemura et al (J Biochem, 79(6):1253-1261, 1976). The Examiner stated that Livingston et al (Cancer Research) teach a composition administered to melanoma patients for stimulation the production of antibodies directed against a carbohydrate epitope on the ganglioside, GM2 (page 7046-7048). The Examiner stated Livingston et al teach that the composition for treatment is administered at a concentrations of

100,200, or 300 ug with an adjuvant, Bacillus-Calmette-Geurin (BCG), and a pharmaceutically acceptable vehicle, phosphate buffered saline (p 7046, column 1, paragraph 3, and paragraph bridging p 7046-47). The Examiner stated Livingston et al teach that melanoma recurrence was delayed in patients developing GM2 antibodies after treatment with the composition (page 7048, paragraph 1 and column 2, paragraph 2). The Examiner stated Livingston et al teach that more patients produced IgM antibodies than IgG antibodies to the GM2, GD2 and GD3 are expressed on the cell surface of human malignant melanomas (page 7045, column 1, paragraph 2). The Examiner stated Livingston et al teach treatment of a melanoma, a cancer which is both epithelial and neuroectodermal in origin. The Examiner stated Livingston et al differ by not teaching the conjugation of the GM2 or other gangliosides by means of a carbon on the ceramide moiety with aminolysyl groups on Keyhole Limpet Hemocyanin (KLH) in a composition and using this composition for treatment. The Examiner stated that Ritter et al (1991) teach that IgG responses to gangliosides may be increased by the covalent attachment of foreign carrier proteins such as KLH to the gangliosides resulting in the T cell help necessary for the response (page 406, paragraph 1). The Examiner stated Ritter et al teaches discloses that the advantage of inducing an IgG antibody response (vs IgM) against gangliosides is that IgG: a) has a higher affinity, b) is better able to penetrate solid tissues, c) is able to mediate antibody-dependent cell-mediated cytotoxicity, d) and is generally detectable in the serum for longer periods after immunization. The Examiner stated Liane et al (Journal of Biological Chemistry, 249(14):4460-4466, 1974) teach a method for covalent coupling of gangliosides to aminoethyl agarose or the amino group bearing glass

beads by oxidative ozonolysis of the olefinic bond of the shpingosine moiety (i.e. the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group of aminoethyl agarose or the amino group bearing glass beads. The Examiner stated Ritter et al (1990) teach that GD3 lactone is more immunogenic than GD3. The Examiner stated Livingston et al (U.S. Patent No. 5,102,663) teach that gangliosides GM3, GM2, GD3, GD2, GT3 and O-acetyl GD3 are gangliosides that are prominent cell-membrane components of melanoma and other tumors of neuroectodermal origin (column 1, lines 22-28). The Examiner stated Kensil et al teach that QS-21 (i.e. the instant carbohydrate derivable from the bark of the Suillaja saponaria Molina tree) produced a higher antibody response than conventional aluminum hydroxide (page 433, column 2, paragraph 4 and Figure 3). The Examiner stated Kensil et al also teach that the immune responses obtained with QS-21, reached a plateau at doses between 10-80 ug in mice (page 433, column 1, paragraph 3). The Examiner stated Maricani et al teach the use of QS-21 adjuvant was useful because it did not cause a toxic reaction in cats (page 93, paragraph 1). The Examiner stated Uemura et al (J Biochem, 79(6):1253-1261,1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity of the ganglioside derivative with antibodies. The Examiner stated it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to modify the composition taught by Livingston et al by conjugating the GM2 to KLH by covalently coupling GM2 to KLH by substituting GM2 for the globoside and KLH for the aminoethyl agarose to produce a GM-2-KLH conjugate by means of the olefinic bond of the sphingosine moiety of the GM2 (i.e. the instant ceramide double bond) and the ε-aminolysyl groups present

in the KLH protein using the method of Liane et al and add QS-21 as an adjuvant to the GM-2-KLH conjugate for use as a vaccine because the conjugated composition would be expected to enhance the IgG response to the ganglioside, as taught by Ritter et al (1991), thus providing the advantages by Ritter et al (1991) and adding the QS-21 would be advantageous because it provides for a higher antibody response that the commonly used adjuvant use by Kensil et al and QS-21 provides the advantages that is not toxic to animals as is taught by Marciani et al. The Examiner stated it also would have been *prima facie* obvious to use doses of between 10 and 80 ug of QS-21 in the composition and optimize the dose accordingly because the immune response with QS-21 plateaus at doses between 10-80 ug and optimization of the weight of ratio of the components of the composition to provide an optimal response is well within the ordinary skill in the art and use the composition as modified supra for treatment of melanoma as taught by Livingston et al (Cancer Research). The Examiner stated it would have been *prima facie* obvious to one of ordinary skill in the art to substitute any one of GM3, GD2, GD3, or O-acetyl GD3 for the GM2 ganglioside in the composition and method as combined supra because they are all prominent cell-membrane components of melanomas as taught by Livingston et al (U.S. Patent No. 5,102,663) and one of ordinary skill in the art at the time the invention was made to substitute the GD3 lactone for the GM2 ganglioside in the composition because GD3 lactone is more immunogenic than GD3, as taught by Ritter et al (1990) and would be expected to product an enhanced antibody response as compared to GD3. The Examiner stated Optimization of the dosage, route to immunization, number of sites of immunization to administer the composition is will within the skill of the ordinary artisan. The Examiner stated one would have reasonably

expected the conjugation procedure to work as substituted because conjugation through the ε-aminolysyl groups of carrier proteins for enhance immunogenicity is routine in the art and Uemura et al (J Biochem, 79(6)1253-1261, 1976) teach that the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies.

In response, applicants respectfully traverse the Examiner's above rejection. Applicants respectfully disagree with the Examiner's contention that the conjugation procedure as combined provides renders obvious the claimed invention. Applicants contend that the cited references, namely Livingston et al. (Cancer Research) in view of Ritter et al. (Seminars in Cancer Biology), Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter et al. (Immunobiol), Kensil et al. (The Journal of Immunology), and Marciani et al. (Vaccine) and Uemura et al (J Biochem) does not teach, suggest or disclose applicants claimed invention and therefore do not render obvious the claimed invention.

Applicants point out that newly amended claim 121 recites a "composition which comprises: a) a conjugate of i) a ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) an immunogenic protein-based carrier; b) a saponin derivable from the bark of a Quillaja saponaria Molina tree; and c) a pharmaceutically acceptable carrier; the relative amounts of such conjugate and such saponin being effective to stimulate or enhance antibody production in a subject, wherein the ganglioside derivative is a derivative of a ganglioside selected from the group

consisting of GM2, GM3, GD2, GD3, GD3 lactone, O-acetyl GD3 and GT3; and wherein the immunogenic protein-based carrier is derived from a protein selected from the group consisting of malaria T-cell epitope, an outer membrane protein of Neisseria meningitidis, cationized bovine serum albumin, Keyhole Limpet Hemocyanin, polylysine and human serum albumin; **wherein in the conjugate the ganglioside derivative is conjugated to the immunogenic protein-based carrier through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative**. "[Emphasis added].

First, the Examiner acknowledges that the primary reference, i.e. Livingston et al. Cancer Research 1989, ("Livingston 1989") does not teach conjugation of GM2 or other gangliosides by means of a carbon on the ceramide moiety (see January 11, 2000 Office Action, page 8).

To compensate for the lack of such disclosure, the Examiner relies relies primarily on two references, namely Ritter et al., Cancer Biology 1991 ("Ritter 1991") and Ritter et al., Immunobiology 1990 ("Ritter 1990"). However, applicants submit that neither of these references supplies what it missing from the primary reference.

Ritter 1991 discloses on page 406, column 1 two approaches for augmenting the immunogenicity of gangliosides in a mouse, and states that only one of these approaches is capable of inducing consistent IgG antibodies to gangliosides in the mouse. Ritter 1991 describes this approach as covalently attaching gangliosides to foreign carrier proteins such as KLH.

Although Ritter 1991 refers to the conjugation of GM2 to KLH, there

is no description of the chemical nature of the conjugate or of how to make the conjugate. Thus, Ritter 1991 neither discloses anything conjugated through the ceramide, nor enables making any such conjugate. Applicants respectfully direct the Examiner's attention to the highlighted portion of claim 97 above relating to the conjugation, which recites "...wherein in the conjugate the ganglioside derivative is conjugated to Keyhole Limpet Hemocyanin through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative to the ε-aminolysyl group of Keyhole Limpet Hemocyanin..." The Examiner's indication that one skilled in the art would interpret Ritter 1991 to involve ceramide conjugation is only speculation. Based on Ritter 1991, one skiiled in the art would not understand that the linkage would be through the ceramide. The Examiner tries to justify that the references teach linkages through the ceramide by using Ritter 1990. However, Ritter 1990 does **not** teach conjugation in a ceramide region.

Ritter 1990 describes making chemical derivatives of GD3. The four derivatives described in Table 1 on page 34 are as follows: (1)amide, which is not immunoreactive with monoclonal antibodies to native GD3; (2) gangliosidol, which is not immunoreactive with monoclonal antibodies to native GD3; (3) lactone I, which is reactive but less than native GD3; and (4)lactone II, which is also reactive but less than native GD3. In Ritter 1990, there is no discussion of a conjugation to KLH. There is merely a description of chemical modifications of the ganglioside. Applicants point out to the Examiner that these derivatives are in the carbohydrate portion and not in the ceramide.

Based on the Table 4 in Ritter 1990, one would interpret that for

GD3, the preference is to make a Lactone 1 derivative, which is a lactone chemically derivatized in the carbohydrate since it is more immunogenic than GD3 itself. Based on Ritter 1990, one would probably make a Lactone derivative. However, there is no suggestion of using the ceramide for such derivative.

The Examiner's attempt to use Ritter 1990 to support his obviousness speculation is incorrect because: (1) Ritter 1990 discloses that the conjugation is through the carbohydrate, not the ceramide; and (2) Ritter 1990 teaches away from ceramide conjugation and indicates that conjugation through a lactone is preferred.

Thus, there is neither a specific disclosure, nor is it obvious from either Ritter 1990 or Ritter 1991 to conjugate through the ceramide. Accordingly, the primary reference (i.e. Livingston et al.) in view of Ritter 1990 and Ritter 1991 does not teach, suggest or disclose the claimed invention Moreover, the other cited references do not supply what is missing from either the primary reference, Ritter 1990 or Ritter 1991.

The Examiner cited Uemura et al as disclosing that ozonolysis and reduction of various sphingolipids do not affect the haptenic activity with antibodies. The Examiner stated that the combination [of references] provides a reasonable expectation of success as demonstrated by Uemura et al which demonstrates the ozonolysis and reduction of various sphingolipids did not affect the haptenic reactivity with antibodies. However, Uemura et al does not supply what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon to a carrier protein.

The Examiner cited Kensil and Marciani for their disclosures with respect to QS-21. The Examiner cited Livingston et al. (U.S. Patent No. 5,102,663) with respect to various gangliosides being cell membrane components of melanoma. Accordingly, neither of these references disclose what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon to a carrier protein.

The Examiner cited Liane et al (Journal of Biological Chemistry), alleging that it "teaches a method for covalent coupling of gangliosides to amino ethyl agarose or the amino group bearing glass beads by oxidative ozonolysis of the olefinic bond of the spingosine moiety (i.e. the instant carbon double bond of ceramide) and coupling of the carboxyl bearing product to the amino group bearing glass beads." Applicants submit that Liane does not supply what is missing from the primary reference with respect to conjugation through a ceramide-derived carbon. In support, applicants attach hereto as **Exhibit A** a copy of Helling et al., Cancer Research 54: 197-203, which was cited as reference 3 in an information disclosure statement filed on May 2, 1997 in connection with the subject application. Applicants point out that Helling et al. addresses the cited reference (i.e. Liane et al.) on page 201, second paragraph of the discussion stating the "earlier" Liane et al. method

> is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Applicants point out that the claimed invention recites in part "...a conjugate of i) a GM2 or GD2 ganglioside derivative which comprises an unaltered oligosaccharide part and an altered ceramide portion comprising a sphingosine base, to ii) Keyhole Limpet Hemocyanin..." Keyhole Limpet Hemocyanin is a carrier protein and accordingly, the Liane et al. methods would not enable a conjugation such as that recited in the claims because the Liane et al. conditions would result in protein degradation. Accordingly Liane et al. does not provide what is missing from the primary reference, i.e. a teaching of a conjugation of a ganglioside to a protein through a ceramide derived carbon, and an enabling disclosure of how to do so. Therefore, the primary reference in view of Liane et al. does not teach, suggest or disclose the claimed invention.

Accordingly, the primary reference, i.e. Livingston 1989 in view of the other cited references, namely Ritter 1990, Liane et al (Journal of Biological Chemistry), Livingston et al. (U.S. Patent No. 5,102,663), Ritter 1991, Kensil et al. (The Journal of Immunology), and Marciani et al. (Vaccine) and Uemura et al (J Biochem) does not render obvious the applicants' claimed invention. Applicants contend that these remarks obviate the above rejection and respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejection under 35 U.S.C. 112, first paragraph

The Examiner rejected claims 71-97 under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one

skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. The Examiner stated this is a new matter rejection. The Examiner stated as to claims 71-97, Applicants point to page 32, lines 1-33 and page 12, lines 4-14 for support for the now claimed invention. The Examiner stated this is not persuasive, the passage at page 32, lines 4-14 provide for a specific coupling procedure at the C-4 carbon of the sphingosine moiety of the ceramide to the ε-aminolysyl group of a proteins (ozonolysis, production of a functional aldehyde group and coupling to an ε-aminolysyl group on a protein by reductive amination). The Examiner stated the passage at page 12, lines 4-14 in combination with the passage at page 32, lines 1-33 does not support a broad coupling to any generic portion of the ceramide backbone of the ganglioside, by any generic means by cleavage of any double bond (i.e. C=O) and coupling by any linkage process. The Examiner stated the written description at pages 12 and 32 does not support by way of written description, convey that applicants had at the time of filing contemplated any means of coupling to any not in possession of that which is now broadly claimed. The Examiner stated correction is required.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have previously canceled claims 57-101 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application, hereinabove canceled claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-121. The newly added

claims recite that the conjugation is through a C-4 carbon of the sphingosine base of the ceramide portion of the ganglioside derivative. Applicants contend that the this language regarding the conjugation obviates the Examiner's above rejection and respectfully request that the examiner reconsider and withdraw this ground of rejection.

The Examiner stated as to claims 89-97, the concept of using the composition for preventing a relapse of cancer in a subject comprising administering the composition is not found in either the detailed description of the invention at pages 11-18, nor at the indicated pages of 12,32,33,76,114 or 116 of the specification as alleged by applicants. The Examiner stated the issue is best resolved by applicants pointing to the specification by page and line number where written description support for conception of this now claimed invention can be found.

In response, applicants without conceding the correctness of the Examiner's position but to expedite prosecution of the subject application have previously canceled claims 57-101 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application, hereinabove canceled claims 102-120 without prejudice or disclaimer to their right to pursue the subject matter of these claims in a later-filed application and added new claims 121-121. The newly added claims do not recite the words "preventing a relapse of cancer." Applicants contend that this amendment obviates the above rejection and respectfully request that the examiner reconsider and withdraw this ground of rejection.

Summary

For the reasons set forth hereinabove, applicants respectfully request that the Examiner reconsider and withdraw the various grounds of objection and rejection and earnestly solicit allowance of the now pending claims, i.e. claims 121-134.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorneys invite the Examiner to telephone them at the number provided below.

No fee, other than the enclosed $945.00 fee for a five month extension of time, is deemed necessary in connection with the filing of this Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of any such fee to Deposit Account No. 03-3125.

Respectfully submitted,

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I hereby certify that this correspondence is being deposited this date with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231.

7-17-01

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[CANCER RESEARCH 54, 197-203, January 1

G_{D3} Vaccines for Melanoma: Superior Immunogenicity of Keyhole Limpet Hemocyanin Conjugate Vaccines[1]

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ABSTRACT

Cell surface gangliosides show altered patterns of expression as a consequence of malignant transformation and have therefore been of interest as potential targets for immunotherapy, including vaccine construction. One obstacle has been that some of the gangliosides that are overexpressed in human cancers are poorly immunogenic in humans. A case in point is G_{D3}, a prominent ganglioside of human malignant melanoma. Using an approach that has been effective in the construction of bacterial carbohydrate vaccines, we have succeeded in increasing the immunogenicity of G_{D3} in the mouse by conjugating the ganglioside with immunogenic carriers. Several conjugation methods were used. The optimal procedure involved ozone cleavage of the double bond of G_{D3} in the ceramide backbone, introducing an aldehyde group, and coupling to aminolysyl groups of proteins by reductive amination. Conjugates were constructed with a synthetic multiple antigenic peptide expressing repeats of a malarial T-cell epitope, outer membrane proteins of *Neisseria meningitidis*, cationized bovine serum albumin, keyhole limpet hemocyanin, and polylysine. Mice immunized with these conjugates showed a stronger antibody response to G_{D3} than mice immunized with unconjugated G_{D3}. The strongest response was observed in mice immunized with the keyhole limpet hemocyanin conjugate of the G_{D3} aldehyde derivative and the adjuvant QS-21. These mice showed not only a long-lasting high-titer IgM response but also a consistent high-titer IgG response (predominantly IgG1), indicating recruitment of T-cell help, although the titers of IgM and IgG antibodies following booster immunizations were not as high as they are in the response to classical T-cell-dependent antigens. This method is applicable to other gangliosides, and it may be useful in the construction of immunogenic ganglioside vaccines for the immunotherapy of human cancers expressing gangliosides on their cell surface.

INTRODUCTION

Gangliosides are glycolipid constituents of the cell membrane. The term was coined in 1942 to refer to lipids of the central nervous system that contained sialic acid, to signify their prime location in ganglion cells and their glycosidic nature (1). Their lipophilic component, the ceramide (an amide-linked long-chain sphingoid base and a fatty acid), is thought to be embedded in the outer membrane of the cell membrane lipid bilayer. The carbohydrate portion of the molecule is oriented toward the outside of the cell. Malignant transformation appears to activate enzymes involved in ganglioside glycosylation, resulting in altered patterns of ganglioside expression in tumors such as astrocytoma, neuroblastoma, and malignant melanoma (2). In normal melanocytes, for example, the predominant ganglioside is G_{M3}.[3] Other gangliosides including G_{D3}, G_{M2}, G_{D1a}, and G_{T1b} constitute less than 10% of the total (3). In malignant melanoma, increased expression of G_{D3}, G_{D2}, and G_{M2} has been observed (4, 5), and these gangliosides have therefore been considered potential targets for immunotherapy.

One approach to ganglioside-targeted immunotherapy has been the use of mAbs.[4] Treatment of patients with melanoma or neuroblastoma with mAb recognizing G_{D3}, G_{D2}, or G_{M2} has resulted in tumor regression in some cases (6–9). The other approach has been to immunize patients with ganglioside vaccines in attempts to induce production of ganglioside antibodies by the patients themselves. These attempts have been successful so far only with G_{M2} vaccines. Patients with American Joint Committee on Cancer Stage III malignant melanoma, after complete resection of all tumor, have been shown to produce anti-G_{M2} antibodies in response to vaccination with G_{M2} and *Bacillus Calmette-Guérin* (after pretreatment with low-dose cyclophosphamide to reduce suppressor activity), and the disease-free interval and overall survival were longer in patients producing G_{M2} antibodies (10). G_{D3} and G_{D2}, on the other hand, were found to be only rarely immunogenic when administered in the same way to patients with melanoma (11). Even with the G_{M2} vaccines, the antibody response showed the characteristics of a T-cell-independent response, that is to say, IgM production of short duration, rare conversion to IgG production, and lack of a booster effect (12, 13).

Similar difficulties have been encountered in the development of effective vaccines against bacterial carbohydrate antigens. One approach that has been successful in overcoming these problems is conjugation of the antigen with immunogenic protein carriers. For example, a conjugate vaccine that links the *Haemophilus influenzae* type b capsular polysaccharide to the outer-membrane protein complex of *Neisseria meningitidis* serogroup B was recently shown to induce the production of antibodies and a high rate of protection against invasive disease caused by *Haemophilus influenzae* type b in infants (14), and similar results were reported for a conjugate vaccine using a nontoxic mutant diphtheria toxin as carrier (15).

We have explored this approach in attempts to increase the immunogenicity of melanoma gangliosides. We report here the effects of conjugating G_{D3} with several protein carriers on its immunogenicity in the mouse.

MATERIALS AND METHODS

Gangliosides. G_{M3}, G_{M2} and G_{D1b}, extracted from bovine brain, were provided by Fidia Research Laboratory (Abano Terme, Italy). G_{D2} was made from G_{D1b} by enzymatic cleavage with β-galactosidase from bovine testes (16). G_{D3} (mel) was isolated from human melanoma tissue (17), G_{D3} (bbm) and GT3 were isolated from bovine buttermilk (18), and disialyllactose (G_{D3} oligosaccharide) was isolated from bovine colostrum as previously described (19).

Reagents. HPTLC silica gel plates were obtained from E. Merck (Darmstadt, Germany); 4-chloro-1-naphthol, *p*-nitrophenyl phosphate disodium, and sodium cyanoborohydride were from Sigma Chemical Co. (St. Louis, MO);

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[2] To whom requests for reprints should be addressed, at Immunology Program, Memorial Sloan-Kettering Cancer Center, 425 East 68th Street, Room 820 K, New York, NY 10021.

[3] The designations G_{M3}, G_{M2}, G_{M1}, G_{D3}, G_{D1a} and G_{D1b} are used in accordance with the abreviated ganglioside nomenclature proposed by Svennerholm (40).

[4] The abbreviations used are: mAb, monoclonal antibody; MAP, multiple antigenic peptide; OMP, outer membrane protein; cBSA, cationized bovine serum albumin; ITLC, immune thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; ELISA, enzyme-linked immunosorbent assays; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline; bbm, bovine buttermilk.

methylsulfide was from Aldrich (Milwaukee, WI); cyclophosphamide (Cytoxan) was from Mead Johnson (Syracuse, NY); and QS-21 adjuvant, a homogeneous saponin component purified from *Quillaja saponaria* Molina tree (22), was kindly donated by Cambridge Biotech Corp (Worcester, MA). It is an amphipathic molecule and was provided as a white powder, forming a clear colorless solution when dissolved in PBS.

Proteins. Poly-L-lysine hydrobromide [MW(vis)3800] was purchased from Sigma, keyhole limpet hemocyanin (KLH) was from Calbiochem (La Jolla, CA), the cBSA-Imject Supercarrier immune modulator was from Pierce (Rockfort, IL), and *Neisseria meningitidis* OMPs were kindly provided by Dr. M. S. Blake (Rockefeller University, New York). MAP YAL-IV 294-I containing four repeats of a malarial T-cell epitope was a gift from Dr. J. P. Tam (Rockefeller University).

Monoclonal Antibodies. Rabbit anti-mouse immunoglobulins conjugated to horseradish peroxidase for ITLC, and rabbit anti-mouse IgM and IgG conjugated to alkaline phosphatase for ELISAs, were obtained from Zymed (San Francisco, CA); anti-G_{D3} mAb R24 was generated in our laboratory (21).

Serological Assays. ELISA were performed as previously described (13). To control for nonspecific "stickiness," immune sera were also tested on plates to which no ganglioside had been added, and the reading was subtracted from the value obtained in the presence of ganglioside. The titer was defined as the highest dilution yielding a corrected absorbance of 0.1 or greater. Immunostaining of gangliosides with mAb or mouse sera was performed after separation on HPTLC silica gel glass plates as previously described (4). Plates were developed in solvent 1 [chloroform:methanol:water (0.25% $CaCl_2$), 50:40:10 (v/v)] or solvent 2 [ethanol:*n*-butylalcohol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v)], and gangliosides were visualized with resorcinol-HCl reagent. Dot-blot immune stains were performed on nitrocellulose strips utilizing purified gangliosides spotted in equal amounts and developed as described before (13).

Immunization. Six-week-old female BALB/c × C57BL/6 F_1 mice (The Jackson Laboratory, Bar Harbor, ME) were given an i.p. injection of cyclophosphamide (15 mg/kg) 3 days before the first immunization and were then assigned to treatment groups. Groups of 4 or 5 mice were given three s.c. injections of a vaccine 2 weeks apart if not otherwise indicated. Each vaccine contained 20 μg G_{D3} or 15 μg disialyllactose and 10 μg QS-21 in a total volume of 0.1 ml PBS. Mice were bled from the retroorbital sinus before vaccination and 2 weeks after the last vaccine injection unless indicated otherwise.

G_{D3} Conjugate Preparation. G_{D3} (2 mg) was dissolved in 2 ml methanol by sonication and cooled to −78°C in an ethanol/dry ice bath. Ozone was generated in an ozone generator (Del Industries, San Luis Obispo, CA) and was passed through the sample for 30 min under vigorous stirring (22, 23). The excess of ozone was then displaced with nitrogen over a period of 10 min. Methylsulfide (100 μl) was added (24), and the sample was kept at −78°C for 30 min and then at room temperature for 90 min under vigorous stirring. The sample was dried under a stream of nitrogen and monitored by HPTLC. The long-chain aldehyde was separated by adding *n*-hexane (2 ml) to the dry sample, followed by sonication for 5 min and centrifugation at 2000 × *g* for 15 min. The *n*-hexane was carefully drawn off and discarded, and the sample was dried under a stream of nitrogen. Cleaved G_{D3} and native G_{D3} were separated by HPLC (Waters, System 501, Milford, MA) utilizing a C_{18} reversed-phase column (10 x 250 mm; Rainin Instruments, Ridgefield, NJ). Gangliosides were eluted with a linear water-acetonitrile gradient and monitored at 214 nm, and the fractions were analyzed by HPTLC. Fractions that contained cleaved G_{D3} were combined and evaporated at 37°C with a Rotavapor (Büchi, Flawils, Switzerland). Cleaved G_{D3} (1.5 mg), 1.5 mg protein carrier in PBS, and 2 mg sodium cyanoborohydride were incubated under gentle agitation at 37°C for 48 h. After 16 h 1 mg sodium cyanoborohydride was added. The progress of coupling was monitored by HPTLC. G_{D3}-protein conjugates did not migrate in solvent 1 and solvent 2 but remained at the origin as a resorcinol-positive band. The mixture was dialyzed across 5000 molecular weight cutoff dialysis tubing with three changes of PBS (4 liters each), at 4°C for 48 h, and passed through an Extractigel detergent-removing gel (Pierce, Rockfort, IL) for final purification of unconjugated G_{D3}. The samples were lyophilized, and their protein and ganglioside content was determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).

Disialyllactose Conjugate Preparation. Disialyllactose was isolated from bovine colostrum as described previously (19). The carbohydrate was attached to protein by reductive amination (26). Disialyllactose (10 mg) was incubated with 2 mg proteins in 2 ml PBS for 14 days at 37°C after sterile filtration. Sodium cyanoborohydride (2 mg) was added at the beginning, and 1 mg was added every 3 days. The coupling was monitored by HPTLC in solvent 2. The disialyllactose conjugates were purified by dialysis across 5000 molecular weight cutoff dialysis membrane with three changes of PBS (4 liters each) at 4°C for 48 h, followed by lyophilization. The protein and neuraminic acid content was determined as described above. Disialyllactose was also conjugated to proteins according to the method described by Roy and Laferrière (27). During this procedure *N*-acroloylated glycopyranosylamine derivatives of the oligosaccharide were formed first, followed by conjugation via Michael addition to amino groups of the protein. Purification and determination of protein and neuraminic acid content were performed as described above.

Determination of Antibody Subclasses. Determination of antibody subclasses was performed by ELISA using subclass-specific rabbit anti-mouse immunoglobulins IgG1, IgG2a, IgG2b, IgG3, and IgA (Zymed, San Francisco, CA). Alkaline phosphatase-labeled goat anti-rabbit IgG served as the signal-generating reagent.

FACS Analysis of Mouse Antisera. A single cell suspension of the melanoma cell line SK-MEL-28 was obtained after treatment with 0.1% EDTA in PBS followed by passage through a 26½-gauge needle. Cells (3×10^5) were incubated with 40 μl of 1:20 diluted post- or preimmunization serum for 30 min on ice. The cells were washed three times with 3% fetal calf serum in PBS. Thirty μl of diluted (1:50) fluorescein isothiocyanate-labeled goat anti-mouse IgG (Southern Biotechnology Associates Inc., Birmingham, AL) were added as secondary antibody, followed by incubation on ice for 30 min. Cells were washed three times as above and resuspended in 500 μl 3% fetal calf serum in PBS and analyzed by flow cytometry (FACScan, Becton Dickinson, San Jose, CA).

RESULTS

Preparation and Characterization of G_{D3}-Protein Conjugates. G_{D3} (bbm) in methanol was selectively cleaved with ozone at the C4-C5 double bond in the ceramide portion. It is assumed that methoxyperoxides are formed as intermediate products (24), and therefore methylsulfide was added as a reducing agent. The result of the cleavage was a G_{D3} derivative with an aldehyde functional group in the position of the former double bond in the ceramide portion (Fig. 1). Cleaved G_{D3} migrated slower than native G_{D3}, and formed double bands because the ceramide contained unsaturated fatty acids that were cleaved simultaneously (see Fig. 1, *inset*). Densitometric analysis of HPTLC plates showed that more than 70% of G_{D3} (bbm) was cleaved by this procedure. Preliminary experiments involving l nger ozone treatment had similar results, indicating that 30% of G_{D3} from this source consists of sphinganine or phytosphingosine analogues that contain no ozone-cleavable ceramide double bond. Cleavage at −78°C with ozone treatment up to 1 h (depending on the amount of G_{D3} used) was found to be optimal. Cleaved G_{D3} persisted only in acidic and neutral phosphate buffers for up to 72 h, but with the formation of increasing amounts of oligosaccharide due to β-elimination reactions [which have been shown to occur much faster at alkaline pH (23)]. The decreased hydrophobicity of cleaved G_{D3} compared to native G_{D3} allowed its separation by HPLC on C_{18} reversed-phase columns. Utilizing isocratic elution with a linear water-acetonitrile gradient, cleaved G_{D3} was recovered first, and uncleaved G_{D3} was eluted in later fractions. The incubation of cleaved G_{D3} with proteins resulted in the formation of Schiff bases between the cleaved ganglioside and ε-aminolysyl groups. They were reduced with sodium cyanoborohydride to form stable secondary amine bonds (28). The reaction was monitored by HPTLC, which showed a decreasing ratio of the cleaved G_{D3} to a resorcinol positive band at the origin, indicating the formation of neoglycoconjugates. The reaction was generally completed after incubation for 48 h at 37°C. Disialyllactose was readily remov-

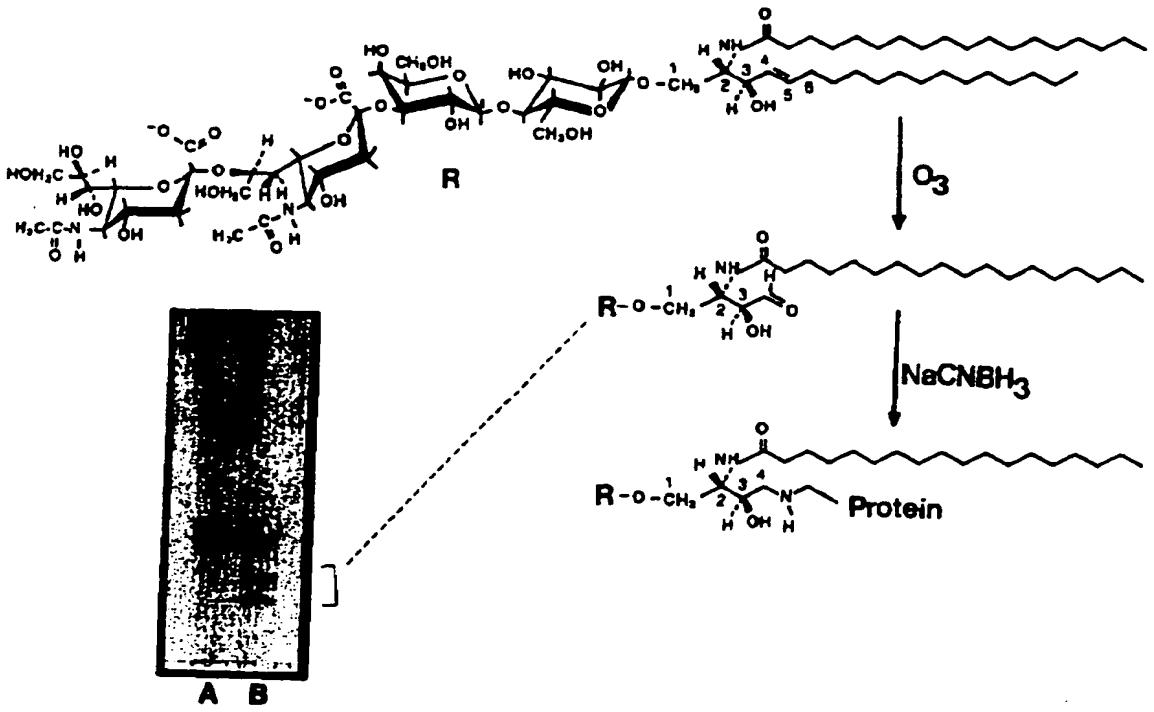

Fig. 1. Synthesis of G_{D3} protein conjugates after ozone cleavage and reductive amination. *Inset*, HPTLC of G_{D3} before (*A*) and after (*B*) ozone cleavage.

able by dialysis, and the excess of cleaved G_{D3} was removed by passage through a detergent-removing column. The degree of coupling was determined by sialic acid and protein determinations. The weight ratio of G_{D3} to proteins in the different conjugates, shown in Table 1, depended on the accessibility of lysine groups in the proteins. The average yield of G_{D3} coupled to proteins was 30%. G_{D3} conjugates prepared in this way were reactive with anti-G_{D3} mAb R24 by Western blot analysis, although the G_{D3}-aldehyde derivative itself was not reactive by ITLC (data not shown).

Oligosaccharide Conjugation. The carbohydrate part of G_{D3}, disialyllactose, was coupled to proteins utilizing two methods. The first method, reductive amination, resulted in conjugation of the open ring form of the glucose to proteins (26). The method required a long incubation of the oligosaccharide with proteins, and the yield was less than 20%. In the second method (27), involving *N*-acroloylation of the terminal glucose, the oligosaccharide was coupled to proteins with a closed ring formation. None of these oligosaccharide conjugates showed reactivity with mAb R24 by Western blot analysis (data not shown).

Induction of a Serological Response against G_{D3} by Immunization with G_{D3}-Protein Conjugates. All vaccines were well tolerated. Mice were observed for at least 6 months, and neither acute nor systemic toxicity was detected. The serological response to immunization with G_{D3} or G_{D3}-protein conjugates, using QS-21 as adjuvant, is shown in Table 1. QS-21 was used because we had previously demonstrated its superiority over other adjuvants with another carbohydrate antigen-KLH conjugate vaccine (29). In ELISA, preimmunization sera showed no IgM or IgG antibodies reactive with G_{D3}. Immunization with unconjugated G_{D3} did not induce the production of G_{D3} antibodies. Immunization with G_{D3} conjugates, on the other hand, was effective in inducing antibody production. Of the five proteins used in the preparation of the conjugates, KLH showed the

Table 1 *Antibody response to immunization with different vaccines containing G_{D3} or disialyllactose conjugated to carrier proteins*

Vaccine + QS-21	No. of mice	G_{D3}:protein weight ratio[a]	Reciprocal ELISA peak titer against G_{D3}	
			IgG	IgM
G_{D3}	5		0 (5)	20 (3), 0 (2)
G_{D3}/KLH[b]	5	0.33	0 (5)	160, 40, 20 (3)
G_{D3}-KLH[c]	14	0.69	10,240 (2), 5,120 (2), 2,560 (3), 1,280 (2), 80 (2), 40 (2), 0	2,560, 1,280 (2), 640, 320 (3), 160 (2), 80 (3), 20, 0
G_{D3}-cBSA[c]	15	0.77	2,560 (2), 320 (2), 160, 80 (2), 40 (4), 20 (2), 0 (2)	80 (2), 40 (2), 20 (7), 0 (4)
G_{D3}-OMP[c]	15	0.93	2,560, 80 (4), 20 (3), 0 (7)	1,280, 320 (2), 160 (7), 80 (4), 40
G_{D3}-MAP[c]	10	1.0	40, 0 (0)	160 (2), 40 (4), 20 (3), 0
G_{D3}-Polylysine	10	ND	0 (10)	320, 160 (4), 80, 40, 20 (2), 0
Disialyllactose-KLH[d]	4	0.055	0 (4)	160 (3), 80
Disialyllactose-cBSA[d]	4	0.16	20, 0 (3)	40, 20 (3)
Disialyllactose-KLH[e]	4	0.25	20, 0 (3)	40 (2), 0 (2)
Disialyllactose-cBSA[e]	4	0.34	0 (4)	0 (4)
Disialyllactose-Polylysine	5	ND	0 (5)	80 (3), 40 (2)

[a] Protein and ganglioside content were determined by BioRad protein assay and by neuraminic acid determination according to the method of Svennerholm (25).
[b] G_{D3} and KLH were mixed prior to immunization.
[c] G_{D3} was covalently attached to proteins prior to immunization after ozonolysis as described in "Materials and Methods."
[d] Disiallyfactose was conjugated to KLH and cBSA by reductive amination according to the method of Gray (26).
[e] Disiallylactose was conjugated to KLH, cBSA, and poly-L-lysine after *N*-acroloylation and Michael addition according to the method of Roy and Lafferière (27).

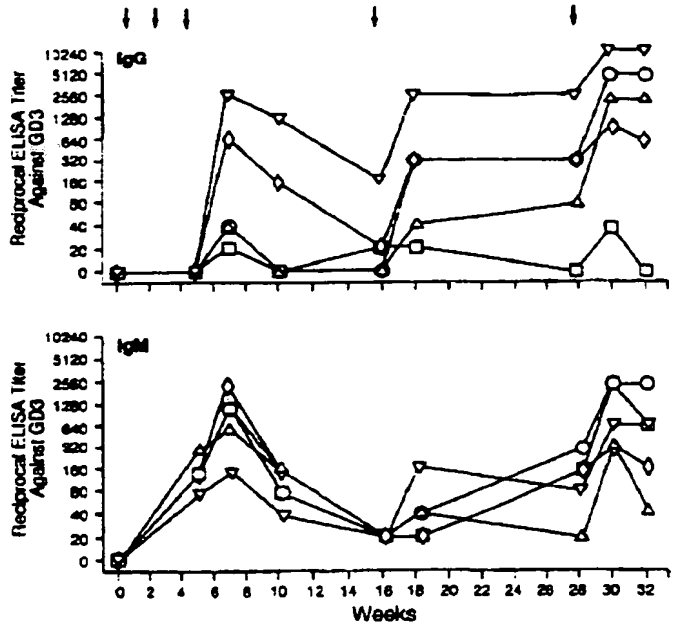

Fig. 2. Time course of G_{D3} antibodies induced in representative mice immunized with G_{D3}-KLH and QS-21 vaccine. Each symbol represents an individual mouse. Arrows, time of vaccination.

strongest immunogenicity, resulting in a median titer of 1:320 for IgM and 1:2560 for IgG antibodies. The specific isotype profile was determined with subclass-specific secondary rabbit anti-mouse antibodies. Antigen-specific antibodies were found to be predominantly of the IgG1 subclass. Antigen-specific IgG2a and IgG2b antibodies were found only in traces, and no IgG3 or IgA antibodies were detected.

In contrast to immunization with G_{D3} conjugates, immunization with G_{D3}-oligosaccharide conjugates induced only a weak IgM response to G_{D3} and no IgG response.

Sequential IgM and IgG antibody titers against G_{D3} for five mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 2. IgM titers peaked 2 weeks after the third vaccination and declined by the time of the first booster immunization at week 16. The first booster immunization had no significant impact on IgM titers, but the second booster immunization at week 28 increased IgM titers to the peak level seen after the third vaccination of the initial series. IgG titers also rose up to 2 weeks after the third vaccination and decreased by the time of the first booster vaccination but rapidly increased after the booster to previous peak titers. IgG titers remained at this level for 10 weeks, with a further increase after the second booster in most mice. The evidence for a secondary immune response after the booster immunization was therefore equivocal. The response was clearly more rapid than after the initial immunization and lasted longer, but the increase in titer was not comparable to booster responses seen with classical T-cell-dependent antigens.

Specificity of the Serological Response to Immunization with G_{D3}-Protein Conjugates. The specificity of the serological response to immunization with G_{D3}-protein conjugates and QS-21 was analyzed by dot-blot immune staining and ITLC. An example of dot-blot immune stain analysis is shown in Fig. 3. Preimmune sera and immune sera showing high G_{D3}-antibody titers in ELISA were tested on nitrocellulose strips that had been spotted with G_{D3} (bbm) or G_{D3} (mel) and purified structurally related gangliosides: G_{M3}, G_{D2}, G_{D1b}, and G_{T3}. As expected on the basis of the ELISA results, preimmune sera showed no reactivity. In contrast, sera obtained after immunization with KLH conjugates of G_{D3}-ganglioside reacted with G_{D3} (bbm) (the immunogen) or G_{D3} (mel), but not with the other gangliosides except G_{T3} in some cases, a pattern also seen in tests of the mouse monoclonal IgG3 antibody R24, the reagent by which high cell surface expression of G_{D3} on human melanoma cells was first defined (20). The same specificity pattern was seen in dot-blot immune stain tests of sera from mice immunized with other G_{D3}-protein conjugates, the only exception being high-titer sera (by ELISA) from mice immunized with G_{D3}-cBSA, which showed no reactivity with G_{D3} or the other gangliosides.

ITLC permits specificity analysis of ganglioside antibodies in tests on tissue extracts. Examples of tests with high-titer sera from mice immunized with G_{D3}-KLH and QS-21 are shown in Fig. 4. The sera were tested at a dilution of 1:150 on ganglioside extracts of human brain, neuroblastoma, and melanoma, as well as G_{D3} (bbm) that had been used for immunization. The figure shows HPTLC ganglioside patterns of these reagents after staining with resorcinol, as compared with the patterns of reactivity exhibited after exposure to sera from immunized mice or mAb R24. As can be seen in the resorcinol-stained panel, the predominant gangliosides in the brain tissue extract are G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b}, whereas the neuroblastoma extract shows G_{D2} and G_{M2} in addition, and the melanoma extract contains mainly

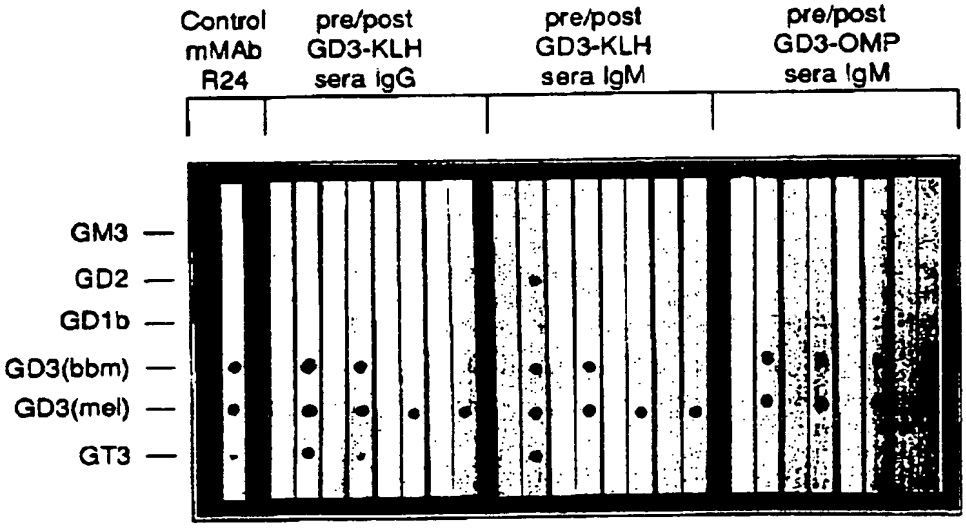

Fig. 3. Dot-blot immune stain assay for IgM and IgG antibodies in sera of mice immunized with G_{D3}-KLH and G_{D3}-OMP conjugates and QS-21. Antigen standards were applied to nitrocellulose strips in equal amounts (0.5 μg) and were allowed to react with pre/postimmunization serum from individual mice.

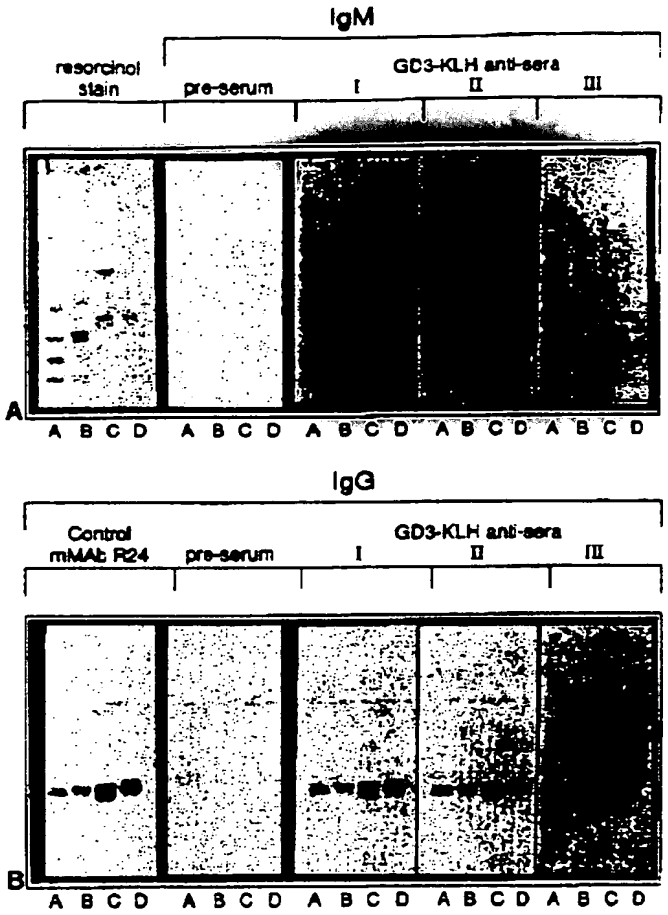

Fig. 4. Immune thin-layer chromatograms of three representative mouse sera after vaccination with G_{D3}-KLH conjugate and QS-21. IgG and IgM antibodies in pre- and postvaccination sera and anti-G_{D3} mMab R24 were tested on human brain gangliosides (*A*), neuroblastoma gangliosides (*B*), melanoma gangliosides (*C*), and G_{D3} (*D*) (bbm). Gangliosides were chemically stained with resorcinol-HCl reagent to demonstrate the ganglioside composition of each sample.

G_{D3} and G_{M3}. Reactivity of IgG antibodies in postimmunization sera, as well the reactivity of IgG3 mouse monoclonal antibody R24, was restricted to G_{D3} (Fig. 4*b*). The high-titer IgM antibodies, on the other hand, showed weak cross-reactivity with other gangliosides and sulfatide in the brain extract (Fig. 4*a*).

Sera from mice immunized with other G_{D3} conjugates were tested in the same way (at lower dilution) and showed the same specificity with the exception, again, of high-titer sera from mice immunized with G_{D3}-cBSA, which showed no ganglioside reactivity (data not shown).

Cell Surface Reactivity of Immune Sera Determined by FACS Analysis. Sera from mice were tested for binding to cells of the melanoma cell line SK-MEL-28, a cell line known to express cell surface G_{D3}. A representative example of a FACS analysis utilizing a fluorescein isothiocyanate-labeled secondary goat anti-mouse antibody is shown in Fig. 5. Sera before and after immunization with G_{D3}-KLH and QS-21 were tested. Preimmunization serum stained 8% of the target cells, postimmunization serum 92%.

DISCUSSION

Conjugation of poorly immunogenic antigens to highly immunogenic carrier molecules is a well-known approach to augmenting immunogenicity. Ganglioside molecules are so small, however, that linkage to carrier molecules without affecting the relevant antigenic epitopes is difficult. We have shown previously that modifications of G_{D3} in its carbohydrate portion (*i.e.*, conversion of sialic acid carboxyl groups to amides or gangliosidels or lactones) results in markedly increased immunogenicity. However, antibodies produced in response to these G_{D3} derivatives show no cross-reactivity with native G_{D3} (11, 30). Covalent attachment of proteins to the sialic acid molecules of G_{D3} was therefore not attempted in the present study. Our initial approach involved conjugation of G_{D3} oligosaccharide (disialyllactose) via the terminal glucose in open- or closed-ring configuration to KLH or polylysine, but these conjugates were not recognized by the anti-G_{D3} mAb R24 or by mouse antisera to G_{D3}, and mice immunized with the conjugates did not produce G_{D3} antibodies. Subsequently, we coupled G_{D3} to proteins via its ceramide portion without alteration of the carbohydrate moiety. The ceramide was cleaved with ozone at the double bond of the sphingosin base, and coupling to proteins was accomplished by reductive amination. Cleavage of gangliosides by ozonolysis and subsequent conjugation with proteins by this method has not been described, and it has been generally assumed that the aldehyde intermediates of gangliosides would be unstable. Fragmentation, initiated by hydroxy ions under alkaline conditions, has been reported. Migration of the double bond would result in β-elimination, causing release of the oligosaccharide moiety (22, 31). We found, however, that the aldehyde was sufficiently stable at neutral pH to permit Schiff base formation with amino groups of proteins, so that β-elimination was not a major problem. The overall yield was 30%. These G_{D3} aldehyde-protein conjugates showed reactivity with G_{D3} antibodies by Western blot analysis, indicating that the immunodominant epitopes were intact in these G_{D3} conjugates. However, reactivity of the G_{D3}-aldehyde derivative with mAb R24 by ITLC could not be shown. This may be due to its relatively unstable nature, resulting in β-elimination and release of oligosaccharide during the immune stain incubation period, or simply to the fact that the G_{D3}-aldehyde derivative may not adhere to the thin-layer plate sufficiently for serological detection.

Earlier studies describe oxidative ozonolysis of the glycosphingolipid olefinic bond, resulting in a carboxyl group that could be conjugated with carbodiimide to NH_2 groups of modified glass beads, agarose gel, or other macromolecules (32, 33). This method, however, is of limited use for the conjugation of gangliosides to carrier proteins because it requires acetylated, methyl ester derivatives of gangliosides to avoid coupling via the sialic acid carboxyl group. Deacetylation after conjugation under basic conditions is necessary, conditions most proteins cannot be exposed to without degradation.

Once the conjugation method was established, several protein carriers were considered, based on previous work by others. Lowell *et al.* (34) described an elegant system that resulted in high-titer antibody responses as a consequence of anchoring bacterial carbohydrate and peptide antigens via a synthetic, hydrophobic foot in OMPs of *Neisseria meningitidis* (35). This system was directly applicable to gangliosides because of their amphipathic nature. In previous studies, we adsorbed gangliosides onto OMP by hydrophobic interaction, and we were able to induce high-titer IgM responses (36). Covalent attachment was utilized in the current study, but G_{D3}-OMP conjugates induced only occasional IgG responses, and the IgM response was not increased. Conjugation with cationized BSA, which has been reported to be a potent carrier for protein antigens (37), resulted in high-titer IgG antibodies detected by ELISA, but immune stains indicated that the response was not G_{D3}-specific. Another appealing carrier is the MAP system described by J. P. Tam (38, 39). MAPs consist of four or eight dendritic peptide arms, containing B- and T-cell epitopes, attached to an oligomeric branched lysine core. The antibody response to peptides was dramatically increased when these constructs were

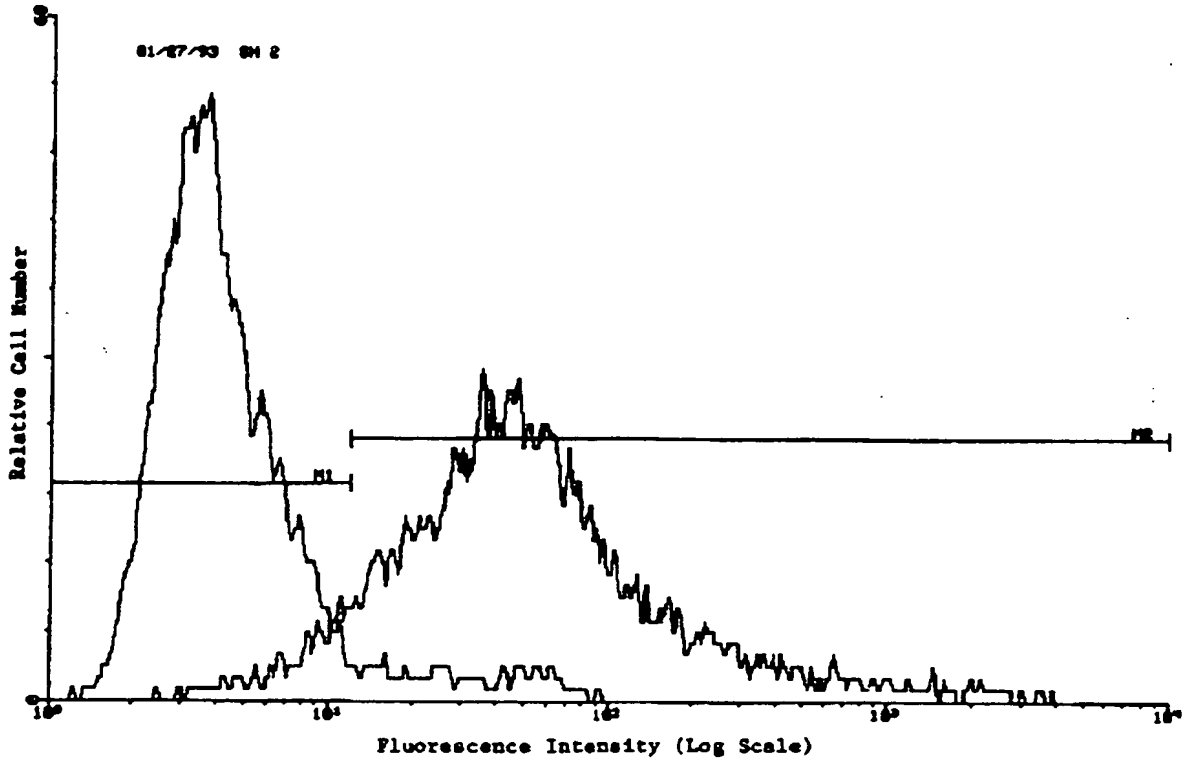

Fig. 5. Representative FACS analysis of mouse serum reactivity prior to (peak at 3) and after (peak at 50) immunization with G_{D3}-KLH and QS-21 tested on melanoma cell line SK-MEL-28.

used. When we attached G_{D3} to the amino terminal end of the MAP structure containing a malarial T-cell epitope, only a moderate IgM response against G_{D3} was detected, and there was no detectable IgG response. Conjugation of G_{D3} to polylysine resulted in a medium-titer IgM response and no IgG response, despite the high density of G_{D3} epitopes on these constructs.

The carrier that proved to be most effective in enhancing the antibody response to G_{D3} in this series was KLH. Immunization with G_{D3}-KLH consistently induced long-lasting production of IgM and IgG antibodies against G_{D3} at high titers. In comparing KLH with cBSA, OMP, MAP, and polylysine, it is difficult to know exactly why KLH is a superior carrier for G_{D3}. The sheer size and antigenic complexity of KLH stand out as a possible aid to antigen processing and recruitment of T-cell help across a broad range of T-cell specificities. The very qualities that make KLH cumbersome to work with are probably responsible for its unique effectiveness as a carrier in conjugate vaccines. KLH has not been widely used as a carrier for conjugate vaccines in humans because its size and heterogeneity make vaccine construction and standardization difficult.

Our hope was that conjugate vaccines would convert the T-cell-independent response against unconjugated G_{D3} seen in our previous studies to a T-cell-dependent response producing high-titer, long-lived, IgG antibodies. This expectation was fulfilled to some extent but not completely. The peak of the IgM response occurred after the third biweekly vaccination as in our previous studies with unconjugated G_{D3}, but the antibody titers were significantly higher. The response declined rapidly (as observed before), and additional vaccinations increased IgM titers to previous peak levels. The repeated increase in the titer of IgM antibodies to G_{D3} after booster immunizations differs from the expected response to T-cell-dependent antigens such as proteins, which generally induce little or no IgM response after booster immunizations. For the first time, however, we were able to induce a high-titer IgG response against G_{D3} ganglioside consistently. This response lasted significantly longer than the IgM response and was increased by additional vaccinations, although the response following booster vaccinations was not comparable to the exponential increase often seen with protein antigens. The fact that the G_{D3} antibodies were of the IgG1 subclass indicates that a T-cell-dependent pathway was activated by the G_{D3}-KLH conjugate vaccine. The lack of a classical booster effect, however, may reflect the carbohydrate nature of G_{D3} and its status as an auto-antigen. This suggests that T-cell recruitment by ganglioside conjugate vaccines is limited by the nature of the antigen itself. Nevertheless, the high-titer IgM response and long-lived IgG response to vaccination with G_{D3}-KLH and QS-21 seen in these experiments represents a striking improvement over the response to unconjugated ganglioside vaccines and can now form the basis for clinical trials of ganglioside-KLH conjugate vaccines in patients with cancers that show increased ganglioside expression.

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REFERENCES

1. Klenk, E. Z. Über die Ganglioside, eine neue Gruppe von zuckerhaltigen Gehirn Lipoiden. Physiol. Chem., *273:* 76–86, 1942.
2. Hakomori, S. I. Aberrant glycosylation in cancer cell membranes as focused on glycolipids: overview and perspectives. Cancer Res., *45:* 2405–2414, 1985.
3. Carubia, J. M., Yu, R. K., Macala, L. J., Kirkwood, J. M., and Varga, J. M. Gangliosides on normal and neoplastic melanocytes. Biochem. Biophys. Res. Commun., *120:* 500–504, 1984.
4. Hamilton, W. B., Helling, F., Lloyd, K. O., and Livingston, P. O. Ganglioside expression on human malignant melanoma assessed by quantitative immune thin layer chromatography. Int. J. Cancer, *53:* 1–8, 1993.

5. Tsuchida, T., Saxton, R. E., Morton, D. L., and Irie, R. F. Gangliosides of human melanoma. *J. Natl. Cancer Inst., 78:* 45–54, 1987.
6. Houghton, A. N., Mintzer, D., Cordon-Cardo, C., Welt, S., Fliegel, B., Vadhan, S., Carswell, E., Melamed, M. R., Oettgen, H. F., and Old, L. J. Mouse monoclonal IgG3 antibody detecting G_{D3} ganglioside: a phase I trial in patients with malignant melanoma. Proc. Natl. Acad. Sci. USA, *82:* 1242–1246, 1985.
7. Cheung, N-K. V., Lazarus, H., Miraldi, F. D., Abramowsky, C. R., Kallick, S., Saarinen, U. M., Spitzer, T., Strandjord, S. E., Coccia, P. F., and Berger, N. A. Ganglioside G_{D2} specific monoclonal antibody 3F8: a phase I study in patients with neuroblastoma and malignant melanoma. J. Clin. Oncol., *5:* 1430–1440, 1987.
8. Irie, R. F., and Morton, D. L. Regression of cutaneous metastatic melanoma by intralesional injection with human monoclonal antibody to ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *83:* 8694–8698, 1986.
9. Irie, R. F., Matsuki, T., and Morton, D. L. Human Monoclonal Antibody to Ganglioside G_{M2} for Melanoma Treatment. The Lancet, 786–787, 1989.
10. Livingston, P. O., Wong, G. Y., Adluri, S., Tao, Y., Padavan, M., Parente, R., Hanlon, C., Calves, M. J., Helling, F., Ritter, G., Oettgen, H. F., and Old, L. J. A randomized trial of adjuvant vaccination with BCG *versus* BCG plus the melanoma ganglioside G_{M2} in AJCC stage III melanoma patients. J. Clin. Oncol., in press, 1994.
11. Ritter, G., Boosfeld, E., Adluri, R., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with ganglioside G_{D3} and G_{D3} congeners (lactones, amide and ganglioside) in patients with malignant melanoma. Int. J. Cancer., *48:* 379–385, 1991.
12. Livingston, P. O., Natoli, E. J. Jr, Calves, M. J., Stockert, E., Oettgen, H. F., and Old, L. J. Vaccines containing purified G_{M2} ganglioside elicit G_{M2} antibodies in melanoma patients. Proc. Natl. Acad. Sci. USA. *84:* 2911–2915, 1987.
13. Livingston, P. O., Ritter, G., Srivastava, P., Padavan, M., Calves, M. J., Oettgen, H. F., and Old, L. J. Characterization of IgG and IgM antibodies induced in melanoma patients by immunization with purified G_{M2} ganglioside. Cancer Res., *49:* 7045–7050, 1989.
14. Eskola, J., Kayhty, H., Takala, A. K., Peltola, H., Ronneberg, P. R., Kha, E., Pekkanen, E., McVerry, P. H., and Makela, P. H. A randomized prospective field trial of a conjugate vaccine in the protection of infants and young children against invasive *Haemophilus influenzae* type b disease. N. Engl. J. Med., *323:* 1381–1387, 1990.
15. Anderson, P. Antibody response to *Haemophilus influenzae* type b and diphteria toxin induced by conjugates of oligosaccharides of the type b capsule with nontoxic protein CRM 197. Infect. Immun., *39:* 233–238, 1983.
16. Cahan, L. D., Irie, R. F., Singh, R., Cassidenti, A., and Paulson, J. C. Identification of a neuroectodermal tumor antigen (OFA-I-2) as ganglioside G_{D2}. Proc. Natl. Acad. Sci. USA, *79:* 7629–7633, 1982.
17. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, F. H., Old, L. J., and Livingston, P. O. Biochemical and serological characteristics of natural 9-*O*-acetyl G_{D3} from human melanoma and bovine buttermilk and chemically *O*-acetylated G_{D3}. Cancer Res., *50:* 1403–1410, 1990.
18. Ren, S., Scarsdale, J. N., Ariga, T., Zhang, Y., Klein, R. A., Hartmann, R., Kushi, Y., Egge, H., Yu, R. K. *O*-Acetylated gangliosides in bovine buttermilk. J. Biol. Chem., *267:* 12632–12638, 1992.
19. v. Nicolai, H., Müller, H. E., und Zilliken, F. Substrate specificity of neuraminidase from *Erysipelothrix rhusiopathiae*. Hoppe-Seyler's Z. Physiol. Chem., *359:* 393–398, 1978.
20. Kensil, C. R., Patel, U., Lennick, M., and Marciani, D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* molina cortex. J. Immunol., *146:* 431–437, 1991.
21. Dippold, W. G., Lloyd, K. O., Li, L. T., Ikeda, H., Oettgen, H. F., and Old, L. J. Cell surface antigens of human malignant melanoma: definition of six antigenic systems with monoclonal antibodies. Proc. Natl. Acad. Sci. USA, *77:* 6114–6118, 1980.
22. Criegee, R. The course of ozonization of unsaturated compounds. Rec. Chem. Prog., *18:* 111–120, 1957.
23. Wiegandt, H., und Baschang, G. Die Gewinnung des Zuckeranteils der Glykosphingolipide durch Ozonolyse und Fragmentierung. Z. Naturforsch., *20b:* 164–166, 1965.
24. Pappas, J. J., Keaveney, W. P., Gancher, E., and Melvin, B. A new and convenient method for converting olefins to aldehydes. Tetrahedron Lett., *36:* 4273–4278, 1966.
25. Svennerholm, L. Quantitative estimation of sialic acids. II. Colorimetric resorcinol-hydrochloric acid method. Biochim. Biophys. Acta., *24:* 604–611, 1957.
26. Gray, G. R. The direct coupling of oligosaccharides to proteins and derivatised gels. Arch. Biochem. Biophys., *163:* 426–428, 1974.
27. Roy, R., and Lafferière, C. A. Michael addition as the key step in the synthesis of sialooligosaccharide protein conjugates from *N*-acroloylated glycopyranosyl-amines. J. Chem. Soc. Chem. Commun., 1709–1711, 1990.
28. Borch, R. F., Bernstein, M. D., and Durst, H. D. The cyanohydridoborate anion as a selective reducing agent. J. Am. Chem. Soc., *93:* 2897–2904, 1971.
29. Livingstone, P. O., Koganty, R. R., Longenecker, B. M., Lloyd, K. O., and Calves, M. J. Studies on the immunogenicity of synthetic and natural Thomsen-Friedenreich (TF) antigens in mice: augmentation of the response by Quil A and SAF-m adjuvants and analysis of the specificity of the responses. Vaccine Res., *1:* 99–109, 1991.
30. Ritter, G., Boosfeld, E., Calves, M. J., Oettgen, H. F., Old, L. J., and Livingston, P. O. Antibody response after immunization with gangliosides G_{D3}, G_{D3} lactones, G_{D3} amide and G_{D3} ganglioside in the mouse. G_{D3} lactone I includes antibodies reactive with human melanoma. Immunobiology, *182:* 32–43, 1990.
31. Kanfer, J. N., and Hakomori, S. Sphingolipid biochemistry. *In:* D. J. Hanahan (ed.), Handbook of Lipid Research, Vol. 3, pp. 49–50. New York: Plenum Press, 1983.
32. Laine, R. A., Yogeeswaran, G., and Hakomori, S-I. Glycosphingolipids covalently linked to agarose gel or glass beads. J. Biol. Chem., *249:* 4460–4466, 1974.
33. Young, W. W., Jr., Laine, R. A., and Hakomori, S. An improved method for the covalent attachment of glycolipids to solid supports and macromolecules. J. Lipid. Res., *20:* 275–278, 1979.
34. Lowell, G. H., Ballou, W. R., Smith, L. F., Wirtz, R. A., Zollinger, W. D., and Hockmeyer, W. T. Proteosome-lipopeptide vaccines: enhancement of immunogenicity for malaria CS peptides. Science (Washington DC), *240:* 800–802, 1988.
35. Donnelly, J. J., Deck, R. R., and Liu, M. A. Adjuvant activity of the outer membrane protein complex of Neisseria meningitidis serogroup B for a polysaccharide-protein conjugate. Vaccines, *91:* 403–408, 1991.
36. Livingston, P. O., Calves, M. J., Helling, F., Zollinger, W. O., Blake, M. S., and Lowell, G. H. G_{D3}/proteosomes vaccine induce consistent IgM antibodies against the ganglioside G_{D3}. Vaccine, *11:* 1199–1204. 1993.
37. Apple, R. J., Domen, P. L., Muckerheide, A., and Michael, J. G. Cationization of protein antigens IV: increased antigen uptake by antigen presenting cells. J. Immunol., *40:* 3290–3295, 1988.
38. Tam, J. P. Synthetic peptide vaccine design: synthesis and properties of a high-density multiple antigenic peptide system. Proc. Natl. Acad. Sci. USA, *85:* 5409–5413, 1988.
39. Tam, J. P., and Lu, Y. Vaccine engineering: Enhancement of immunogenicity of synthetic peptide vaccines related to hepatitis in chemically defined models consisting of T- and B-cell epitopes. Proc. Natl. Acad. Sci. USA. *86:* 9084–9088, 1989.
40. Svennerholm, L. Chromatographic separation of human brain gangliosides. J. Neurochem., *10:* 613–623, 1963.